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NOTE

Molecular identification and virulence of three *Aeromonas hydrophila* isolates cultured from infected channel catfish during a disease outbreak in west Alabama (USA) in 2009

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ABSTRACT: Three isolates (AL09-71, AL09-72, and AL09-73) of *Aeromonas hydrophila* were cultured from infected channel catfish *Ictalurus punctatus* during a disease outbreak in west Alabama, USA, in August 2009. Sequence analysis of the 16S–23S rDNA intergenic spacer region (ISR), *cpn60, gyrB*, and *rpoD* genes of the 3 strains revealed that the 3 strains were closely related to each other, sharing 97 to 99% nucleotide sequence similarities. However, ISR sequences of the 3 isolates from 2009 shared only 64% nucleotide sequences with AL98-C1B, a 1998 isolate of *A. hydrophila* cultured from diseased fish in Alabama. Sequences of *cpn60, gyrB,* and *rpoD* from the 3 isolates from 2009 shared 91 to 95% homologies with AL98-C1B. Based on both LD_{50} and LD_{95} values of intraperitoneal injection assays, the virulences of the 3 isolates from 2009 were not significantly different from each other, but were at least 200-fold more virulent than AL98-C1B, indicating that the 3 west Alabama isolates of *A. hydrophila* from 2009 were highly virulent to channel catfish.

KEY WORDS: *Aeromonas hydrophila* · Molecular analysis · Virulence · Channel catfish

INTRODUCTION

Aeromonas hydrophila, a Gram-negative motile bacillus widely distributed in aquatic environments, is a causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al. 2003), also known as epizootic ulcerative syndrome (EUS) (Mastan & Qureshi 2001). The symptoms of *A. hydrophila* infections include swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia (Karunasagar et al. 1989, Azad et al. 2001). Fish species affected by MAS include tilapia (Abd-El-Rhman 2009, Tellez-Bañuelos et al. 2010), catfish (Majumdar et al. 2007, Ullal et al. 2008), goldfish (Irianto et al. 2003, Harikrishnan et al. 2009), common carp (Jeney et al. 2009, Yin et al. 2009), and eel (Esteve et al. 1994). Although usually considered as a secondary pathogen associated with disease outbreaks, *A. hydrophila* could also become a primary pathogen in some environments, causing outbreaks in fish farms with high mortality rates and severe economic losses to the aquaculture industry worldwide (Thorpe & Roberts 1972, Nielsen et al. 2001, Fang et al. 2004).

Between June and October 2009, a disease outbreak occurred in 48 catfish farms in west Alabama, USA, causing an estimated loss of more than 3 million pounds (ca. 1339 metric tons) of food size channel catfish *Ictalurus punctatus* (Hemstreet 2010). The disease produced a variety of symptoms that included sores on the skin, bulging eyes, ulcers, and bright red muscles and internal organs. Bacteria were cultured from diseased catfish, isolated, and later reported to be *Aeromonas*

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hydrophila (Hemstreet 2010). However, identification evidence was not shown in that short report.

Sequencing of the 16S–23S rDNA intergenic spacer region (ISR) is now considered a robust and sensitive taxonomic tool which is widely used in bacterial taxonomy (Martínez-Murcia et al. 2005, Tazumi et al. 2009). Gene sequences of 60 kDa chaperonin (*cpn60*), DNA gyrase B subunit (*gyrB*), and RNA polymerase sigma factor (*RpoD*/*rpoD*) have also been used in previous studies to identify *Aeromonas* species (Miñana-Galbis et al. 2010). Three *A. hydrophila* isolates from 2009 and isolate AL98-C1B (collected in Alabama in 1998) were compared at the molecular level using the aforementioned 4 genes. The virulence of all 4 isolates against channel catfish was also compared.

MATERIALS AND METHODS

Bacterial strain isolation, identification, and growth conditions. Three isolates of *Aeromonas hydrophila* were collected from diseased food size channel catfish in August 2009 from west Alabama, USA. *A. hydrophila* isolate AL98-C1B was isolated from diseased channel catfish in Alabama in 1998. All 4 isolates were cultured on tryptic soy agar (TSA) plates according to published procedures (Panangala et al. 2007). Isolates of *A. hydrophila* were then determined by standard biochemical tests as described by Holt et al. (1994) and confirmed by API 20 E strip tests (BioMerieux USA) and fatty acid methyl ester analysis by the MIDI microbial identification gas chromatography system. All 4 isolates were maintained on TSA plates or in tryptic soy broth (TSB, Difco) at 28°C for 18 to 24 h.

Genomic DNA extraction and polymerase chain reaction (PCR). Genomic DNA was extracted from pure bacterial cells using a DNeasy Kit (Qiagen) and quantified on a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). Gene-specific primers were then designed to amplify regions of 4 genes: 16S–23S rDNA ISR, *cpn60*, *gyrB*, and *rpoD*. Primer sequences used in PCR are listed in Table 1. PCR was performed in a 10 µl mixture consisting of 5 µl of *Taq* PCR Master Mix (Qiagen), 3 µl of nuclease-free H_2O, 1 µl of *Aeromonas hydrophila* genomic DNA (10 ng μl^{-1}), 0.5 µl of forward primer (5 µM), and 0.5 µl of reverse primer (5 µM). All PCRs were carried out in a Biometra T Gradient thermocycler. The PCR reaction conditions consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C and maintained at 4°C.

Sequencing and sequence analysis. PCR products were verified by electrophoresis on a 1% agarose gel stained with ethidium bromide. PCR products were then purified using a PCR DNA purification kit (GE health care) according to the manufacturer's protocol and sent to USDA-ARS Mid South Genomic Laboratory (Stoneville, MS) for sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems). The amplification primers listed in Table 1 were used for the forward and reverse sequencing. Sequences were analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies.

Virulence of 4 *Aeromonas hydrophila* isolates to channel catfish. All 4 *A. hydrophila* isolates were grown in TSB (Difco) at 28°C for 18 to 24 h. The concentration (colony forming units [CFU] ml^{-1}) of *A. hydrophila* used in this study was determined through serial dilutions. An optical density (OD) of 1.0 of the bacterial cultures was measured at 540 nm using a thermospectronic spectrophotometer (Fisher Scientific). At least 5 different dilutions of the OD = 1.0 overnight bacterial culture for each isolate was used to inject fish. The OD = 1.0 bacterial cultures were then stored at 4°C for later plate counting. Soon after the fish were injected by different dilutions of bacteria (<1 h), serial dilutions (in triplicate) of each *A. hydrophila* isolate were prepared in TSB, and 100 µl of each dilution were plated onto TSA plates. After 24 h incubation at 28°C, the average number of CFU ml^{-1} was

Table 1. Primers used in PCR amplification and sequencing of 16S-23S rDNA ISR (16S ISR), 60 kDa chaperonin (*cpn60*), DNA gyrase B subunit (*gyrB*), and RNA polymerase sigma factor (*rpoD*) *genes*

Gene	Accession no.	Primer name	Primer sequence (5′–3′)	Melting temp. (°C)
16S ISR	NC_008570	16S-Forward	CACGGTGTGATTCATGACTGG	66.50
		23S-Reverse	AAGGCATCCACCATGTACGC	67.00
cpn60	NC_008570	cpn60-Forward	AAGATGCTGGAAGGCGTAAA	63.50
		cpn60-Reverse	GTTGACGAAGTACGGGGAGA	64.00
gyrB	NC_008570	gyrB-Forward	TACCCTGCTGCTGACCTTCT	63.90
		gyrB-Reverse	AAGTCGTAGCTGAGCGGGTA	63.70
rpoD	NC_008570	rpoD-Forward	GTGGTCTGCAGTTCCTGGAT	64.10
		rpoD-Reverse	ATACGCTCACGGGTAACGTC	63.80

calculated for each isolate, and the different amounts of bacteria injected into fish in each treatment group were calculated according to their dilution factor. At least 5 different amounts (ranging from 1×10^4 to 2×10^9 CFU fish^{-1}) of each isolate that kills 0 to 100% fish were administered to channel catfish (industry pool strain) though intraperitoneal (IP) injection. Channel catfish (26.5 ± 4.5 g) naïve to *A. hydrophila* exposure were randomly obtained from stocks maintained at the USDA-ARS-Aquatic Animal Health Research Unit at Auburn, Alabama, and acclimated for 7 d prior to challenge. Acclimated fish were maintained in 57 l glass aquaria with flow-through (0.5 l min^{-1}) dechlorinated tap water and constant aeration with water temperature at 28°C. The dissolved oxygen range was between 6 and 8 mg l^{-1}. A 12:12 h light:dark cycle was maintained. Fish were fed daily with commercial Aquamax Grower at 4% of their body weight. After exposing catfish to *A. hydrophila*, mortalities were recorded daily for 14 d post exposure. The presence or absence of *A. hydrophila* in dead fish was determined by culturing anterior kidney samples on blood agar plates followed by biochemical analysis. Lethal doses that caused 50% mortality (LD_{50}) were calculated using PoloPlus probit and logit analysis software (LeOra Software). Virulence between different isolates was considered significantly different when the 95% confidence intervals of LD_{50} values failed to overlap ($p \leq 0.05$).

RESULTS AND DISCUSSION

API 20E biochemical identification results of the 4 bacterial isolates revealed that all 4 isolates were *Aeromonas hydrophila*, which shared similar biochemical profiles as known *A. hydrophila* deposited into the Mini API strip reading system (Biomerieux). The 3 isolates from 2009 were positive for β-galactosidase, arginine dihydrolase, citrate utilization, indole production, acetoin production, gelatinase, glucose oxidation, mannitol oxidation, inositol oxidation, saccharose oxidation, arabinose oxidation, and cytochrome oxidase; they were negative for lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, tryptophane deaminase, sorbitol oxidation, rhamnose oxidation, melibiose oxidation, and amygdalin oxidation. The 1998 Alabama isolate AL98-C1B had the same biochemical profile in the API 20E strip test, except that its reading of inositol oxidation and arabinose oxidation were both negative. Fatty acid methyl ester analysis by the MIDI microbial identification gas chromatography system revealed that all 3 isolates from 2009 had high similarity indices (0.682, 0.690, 0.724, respectively, for AL09-71, AL09-72, AL09-73) with *A. hydrophila* deposited in the RCLN50 database of the microbial identification system. AL98-C1B had a lower similarity index (0.380) with *A. hydrophila* deposited in the RCLN50 database, suggesting that AL98-C1B is distantly related to the 3 isolates from 2009.

Sequencing results of PCR products obtained for 16S–23S ISR, *cpn60*, *gyrB*, and *rpoD* from the 4 *Aeromonas hydrophila* isolates were deposited in GenBank under accession numbers HM856359 to HM856374. Comparing the 2009 isolates and the 1998 AL98-C1B isolate with *A. hydrophila* ssp. *hydrophila* ATCC7966 (accession no. NC_008570), the 2009 isolates shared the highest homology with ATCC7966 (with homologies ranging from 94 to 95% with 16S–23S ISR, 96 to 98% with *cpn60*, 96% with *gyrB*, and 97% with *rpoD*). Sequences of AL98-C1B shared lower homology with ATCC7966 (63% with 16S–23S ISR, 91% with *cpn60*, 93% with *gyrB*, and 95% with *rpoD*). The 3 Alabama isolates from 2009 shared high homology with each other (16S–23S ISR shared 97 to 98% homology, *cpn60* shared 98 to 99%, and *gyrB* or *rpoD* shared 99%); however, they shared lower homology with AL98-C1B (63 to 64% with 16S–23S ISR, 90 to 91% with *cpn60*, 92% with *gyrB*, and 95% with *rpoD*). Pairwise homologies are given in Table 2.

Mortalities were observed as early as 6 h post injection at a dose of 1.6×10^5 CFU fish^{-1} or higher following IP injection of channel catfish fingerlings with the 3 isolates from 2009, and the majority of the mortalities occurred within 24 h post injection. Cultures from dead fish were all confirmed to be *Aeromonas hydrophila*. The 2009 isolates were significantly ($p < 0.05$) more virulent than the 1998 isolate AL98-C1B based on both LD_{50} and LD_{95} values (Table 3). Based on LD_{50} values,

Table 2. *Aeromonas hydrophila.* Homology (%) between sequences of different isolates. Full gene names as in Table 1

Gene	Isolate	AL09-71	AL09-72	AL09-73	AL98-C1B
16S ISR	AL09-72	97			
	AL09-73	98	98		
	AL98-C1B	63	64	63	
	ATCC7966	94	95	95	63
cpn60	AL09-72	99			
	AL09-73	98	98		
	AL98-C1B	91	91	90	
	ATCC7966	98	98	96	91
gyrB	AL09-72	99			
	AL09-73	99	99		
	AL98-C1B	92	92	92	
	ATCC7966	96	96	96	93
rpoD	AL09-72	99			
	AL09-73	99	99		
	AL98-C1B	95	95	95	
	ATCC7966	97	97	97	95

Table 3. *Aeromonas hydrophila.* LD_{50} and LD_{95} values (in colony forming units per fish) of the 4 isolates to channel catfish by intraperitoneal (IP) injection. Different letters within columns indicate that the virulences of the *A. hydrophila* strains were significantly different from each other because the 95% confidence intervals (CI) failed to overlap

Strain	LD_{50} (95% CI)	LD_{95} (95% CI)	Slope (SE)	χ^2
AL09-71	1.1×10^5 (6.2×10^4–2.1×10^5)^A	7.0×10^5 (3.1×10^5–2.1×10^7)^A	3.04 (0.68)	1.11
AL09-72	1.9×10^5 (1.3×10^5–2.5×10^5)^A	4.8×10^5 (4.8×10^5–1.8×10^6)^A	5.11 (0.53)	2.49
AL09-73	1.3×10^5 (8.9×10^4–1.8×10^5)^A	4.8×10^5 (3.0×10^5–1.8×10^6)^A	3.76 (0.78)	0.47
AL98-C1B	2.3×10^7 (1.2×10^7–4.2×10^7)^B	2.0×10^8 (9.1×10^7–1.6×10^9)^B	3.03 (0.49)	1.30

AL09-71 was the most virulent 2009 isolate, followed by AL09-73 and AL09-72 (Table 3). However, the difference in virulence among the 3 isolates was not significantly different.

Precise identification of pathogen is a prerequisite to successful control of disease outbreak. Identification of *Aeromonas* at the species level by routine procedures involves many difficulties because of the absence of a unified identification key and the lack of agreement between biochemical and genetic identification schemes (Soler et al. 2004, Ormen et al. 2005). Using biochemical identification methods, we were able to identify the 3 isolates from 2009 as *A. hydrophila* and confirmed that they differed from AL98-C1B, but we were unable to determine whether the 2009 isolates were the same or different by biochemical methods. Sequencing of the 16S–23S rDNA ISR is considered a robust and sensitive taxonomic tool which is widely used in bacterial taxonomy (Martínez-Murcia et al. 2005, Tazumi et al. 2009). Sequence comparisons based on 16S–23S rDNA ISRs of the 4 isolates revealed that the 2009 isolates were closely related to each other, but very distantly related to AL98-C1B, suggesting that AL98-C1B and the 3 more recent isolates were different strains. Sequences of *cpn60* of the 4 isolates shared higher homology (91 to 98%) with *cpn60* of the ATCC7966 strain than sequences of ISR of the 4 isolates (63 to 95% homology with ATCC7966), suggesting that ISR sequences of different *A. hydrophila* were more divergent than *cpn60* sequences and that ISR might be a better taxonomic tool to differentiate closely related *Aeromonas* species. Nonetheless, both ISR and *cpn60* sequences revealed the same relationships among the 4 isolates, suggesting that both ISR and *cpn60* were appropriate in differentiating the 4 isolates from Alabama.

The *gyrB* and *rpoD* sequences of the 2009 isolates also differentiated from the AL98-C1B isolate (Table 2). However, among the 2009 isolates, *gyrB* and *rpoD* shared 99% homology, suggesting that both *gyrB* and *rpoD* are very conserved house-keeping genes, which might not be more appropriate molecular markers to differentiate closely related *Aeromonas* species.

Our data suggested that the 2009 isolates were highly virulent to channel catfish, which might explain why so many fish were killed in the 2009 disease outbreak in west Alabama. Virulence studies of the 4 isolates revealed that most infected fish died within 24 h post exposure, suggesting that rapid-acting virulence factors might play an important role in the disease. The identities of the virulence factors and their exact roles in *Aeromonas* disease merit further study.

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